Fish and Shellfish Immunology 87 (2019) 609–614

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Immuno-stimulatory effects of sulfated polysaccharides isolated from *Codium fragile* in olive flounder, *Paralichthys olivaceus*

Yong Yang[a], Jinhwan Park[b], Sang Guan You[c], Suhee Hong[a,b,*]

[a] Department of Marine Biotechnology, Gangneung-Wonju National University, Gangneung, 210-702, South Korea
[b] Department of Wellness Bio-Industrial, Gangneung Wonju National University, Gangneung, 210-702, South Korea
[c] Department of Marine Food Science and Technology, Gangneung-Wonju National University, Gangneung, 210-702, South Korea

ARTICLE INFO

Keywords:
Olive flounder
Codium fragile
Sulfated polysaccharides
Immune gene
Lysozyme
Complement

ABSTRACT

Sulfated polysaccharides isolated from *Codium fragile* have been previously demonstrated to possess immune-stimulating effects on murine cell lines and the fraction F2 (F2) isolated by ion exchange chromatography was the most effective. In this study, the effects of the fraction F2 were evaluated on the expressions of immune genes including IL-1β, TNF-α, IL-8, IFN-γ and lysozyme *in vitro* and *in vivo* as well as lysozyme and complement activities in serum of olive flounder, *Paralichthys olivaceus*. *In vitro*, these gene expressions were up-regulated by F2 in head kidney cells. *In vivo*, IL-1β and IL-8 gene expressions were up-regulated in peritoneal cells, head kidney, liver, gill and spleen, while TNF-α, IFN-γ and lysozyme gene expressions were mostly up-regulated but varied depending on tissue types or time points. Indeed, lysozyme and complement activities in serum were increased. Overall, these results indicate that the sulfated polysaccharides from *C. fragile* have immuno-stimulatory effects on olive flounder and may be used to enhance immunity during aquaculture.

1. Introduction

Olive flounder, *Paralichthys olivaceus* is abundantly raised in Korea, Japan and China [1]. During an intensive aquaculture, olive flounder is exposed to a high risk of infection like other cultured fishes. Among conventional disease controlling methods, antibiotics have problems of resistance and residues and vaccination limits its use to only specific diseases. To overcome these disadvantages, enhancing nonspecific protective immunity of fish by diet is feasible to promote healthy and sustainable aquaculture since diet and immunity are closely related [2].

Sulfated polysaccharides (SPs) are anionic macromolecules that contain sulfates. The SPs from marine algae are known to have various physiological functions including anti-inflammatory, anti-tumor, anti-viral and immune modulating activities [3–5]. *Codium fragile* is a dark green alga distributed in Asia, Europe, Mediterranean Sea and America [6]. Tabarsa et al. [7] found that the SPs from *C. fragile* could activate NF-κB/MAPK pathways in murine macrophage cell line (RAW264.7) to produce inflammatory cytokines and the second fraction (F2) of the SPs fractionated by ion exchange is the most immune-stimulating part. Surayot et al. [8] also demonstrated that F2 could induce inflammatory cytokine gene expressions in natural killer (NK) cells through CR3 pathway. Our previous study also revealed that F2 had a stimulatory effect on IL-1β gene expression in head kidney (HK) cells of olive flounder [9].

In the present study, we have expanded our study to assess the immuno-stimulatory effects of the F2 on immune genes in immune tissues to be used as feed additive to enhance fish immunity in the future. For this, we analyzed the expression of immune genes including IL-1β, IL-8, TNF-α, IFN-γ and lysozyme. IL-1β and TNF-α are fundamental pro-inflammatory cytokines; IL-1β was the first cytokine characterized in fish [10]. IL-8 is a typical chemokine which mediates chemotaxis and activation of neutrophils [11]. IFN-γ is the sole member of type II interferon and plays an essential role in both innate and adaptive immunity [12]. Lysozyme catalyzes the hydrolysis of peptidoglycan in bacterial cell wall and can be served as an indicator of the innate immunity [13]. There are 2 types of lysozymes in Osteichthyes, i.e., g-type and c-type, and usually the c-type lysozyme is more dominant [14,15].

Therefore, in this study, pro-inflammatory cytokines (IL-1β and TNF-α), chemokine (IL-8), type II interferon (IFN-γ) and c-type lysozyme gene expressions were analyzed to elucidate the immuno-stimulatory effects of SPs from *C. fragile in vitro* and *in vivo* in olive flounder. Besides, the activity of lysozyme and complement in serum was also studied.

* Corresponding author. Department of Marine Biotechnology, Gangneung Wonju National University, Gangneung, 210-702, South Korea.
E-mail address: s.hong@gwnu.ac.kr (S. Hong).

https://doi.org/10.1016/j.fsi.2019.02.002
Received 24 October 2018; Received in revised form 28 January 2019; Accepted 1 February 2019
Available online 02 February 2019
1050-4648/

Table 1
Oligonucleotide primers used in qRT-PCR analysis.

Genes	Primers	Sequences (5' - 3')	Sizes	Accession Nos.
Elongation factor-1α	EF-1α F	CTCCACTGAGCCCCCTTACA	235 bp	AB017183
	EF-1α R	GTCTCCGTGCCAACCAGAGA		
Interleukin-1β	IL-1β F	GACAGTGAGATGGTGCGATTTC	128 bp	AB070835
	IL-1β R	ACCATCACTGGCCTGTTGTCT		
Interleukin-8	IL-8 F	CCTCTCTGGCCATCAGTGAAG	135 bp	AF216646
	IL-8 R	AGTGAGAGTTGGGAGAGATTATTTCC		
Tumor necrosis factor-α	TNF-α F	AGGAGGCAGCGGAAAAACA	130 bp	AB040448
	TNF-α R	TAGGCGTCCTCCTGACTCTTCT		
Interferon-γ	IFN-γ F	CTGTCTGTCCCTGTGTCTTTGTG	130 bp	AB435094
	IFN-γ R	GGGCTTCCCGTTGAATCTGT		
Lysozyme	Lysozyme F	GATCCACTGACTACGGCATCTTC	130 bp	AB050469
	Lysozyme R	TTGATCGCCACAATGACATCA		

2. Materials and methods

2.1. Polysaccharides

The *C. fragile* was collected from the coast of Sokcho, Korea, extracted SPs by distilled water at 65 °C and then fractionated by ion-exchange chromatography. Three fractions (F1, F2 and F3) were yielded and the F2 fraction was chosen to assess its immuno-stimulating effects [7]. The lyophilized F2 was dissolved in 0.01M PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH = 7.6) with a concentration of 2 mg/mL, autoclaved and stored at −20 °C before use. The bioactivity of F2 was not obvious affected in the following experiment after autoclaving.

2.2. Fish

Healthy olive flounder (around 10 g) were purchased from a fish farm of Geoje island located in southern part of Korea and kept at approximately 18 ± 1.2 °C in a circular 200 L tank continuously supplied with seawater in the Marine Biology Center for Research and Education at Gangneung-Wonju National University, Korea. The fish was fed daily with commercial diet at a ratio of 0.8% of body weight.

2.3. In vitro analysis

HKs were aseptically taken from four healthy fish after anesthetized with 0.3‰ 2-phenoxy ethanol (Sigma-Aldrich), passed through nylon mesh (pore size 100 μm, BD Biosciences, USA) and washed twice with L15 medium (Gibco, USA) containing 2 × Antibiotic-Antimycotic (Gibco). The cells were adjusted to 1×10^6 cells/mL with L15 containing 1 × Antibiotic-Antimycotic, added 1 mL into a well of 12-well plates and incubated with 0 (control), 0.2, 1 or 5 μg/mL of F2 for 4, 8, 24 h at 20 °C. Then the cells were lysed with QIAzol (Qiagen, Germany) after removing medium and stored at −80 °C until RNA extraction.

2.4. In vivo analysis

Sixty-four healthy fish (around 10 g) was randomly divided into 4 groups and intraperitoneally (i.p.) injected with 0, 4, 20, or 100 μg of F2 after anesthetization with 0.3‰ 2-phenoxy ethanol. Eight fish in each group was sacrificed at day 1 or 3 post-injection by overdosed anesthetization and cutting spinal cord. Blood was collected from caudal vein, clotted for 3 h at 4 °C, and centrifuged at 800 × *g* for 10 min to obtain serum. Peritoneal cells (PC) were collected by i.p. injection of 200 μL of PBS, recollecting the liquid and centrifuging at 800 × *g* for 10 min. HK, liver, gill, and spleen were aseptically taken, immersed into 1 mL of QIAzol and immediately frozen in liquid nitrogen. The samples were stored at −80 °C until used. The experiments on fish complied with the Guide for the Care and Use of Laboratory Animals, 8th edition and approved by Gangneung-Wonju National University.

2.5. Total RNA extraction and cDNA synthesis

Total RNA was isolated using QIAzol following the manufacturer's instructions and dissolved in nuclease-free water (Gibco). Around 1 μg of total RNA was reverse-transcribed into complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), diluted with 480 μL of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0) and stored at −20 °C.

2.6. Quantitative real-time PCR analysis

To analyze immune gene expression, quantitative real-time PCR (qRT-PCR) was performed using LightCycler 96 (Roche, Switzerland) in duplicate for each sample. The qRT-PCR was performed in a 20 μL reaction volume containing 10 μL of SYBR Green Real-time PCR Master Mix (TaKaRa, Japan), 2 μL of primer mix containing 5 μM forward and reverse primers (Table 1), 4 μL of cDNA and 4 μL of nuclease-free water. The protocol was as follows: initial-denaturation for 600 s at 95 °C; amplifying for 45 cycles with denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C; reading the plate at 75 °C. Serially diluted references were used for absolute quantification analysis. After normalizing to the expression level of housekeeping gene, i.e., EF-1α, fold changes were calculated by dividing the normalized ratio by control group at each time point.

2.7. Humoral assay

Lysozyme activity in serum was assessed using EnzChek Lysozyme Assay Kit (Life technologies, USA). Briefly, 5 μL of serum was diluted with 45 μL of 1 × reaction buffer and incubated with 50 μL of fluorescein labeled *Micrococcus lysodeikticus* (50 μg/mL) for 2 h at 37 °C. The fluorescence was measured with the excitation/emission wavelengths of 485/535 nm in Mithras LB 940 (Berthold Technologies, Germany). The lysozyme activity was calculated from the standard curve made using serially diluted chicken egg white lysozyme.

The complement activity (alternative pathway) assay was performed following Yano et al. [16] with some modifications. Briefly, sheep red blood cells (SRBC, Fitzgerald, USA) was washed 3 times and adjusted to 2×10^8 cell/mL in EGTA-Mg-GVB (10 mM EGTA, 10 mM Mg^{2+}, 5 mM sodium barbiturate, 145 mM NaCl, 0.1% (w/v) gelatin, pH 7.5). Serially diluted serum (100 μL) were added to 40 μL SRBC in the 96-well plate, incubated in shaking incubator with a speed of 60 × rpm for 90 min at 25 °C and then centrifuged at 800 × *g* for 5 min at 4 °C. Eighty μL supernatants were transferred to a well of 96-well plate to check the absorbance at 405 nm in a spectrophotometer (Biotek, USA). The reciprocal of plasma dilution causing 50% lysis of SRBC was deemed as ACH_{50} [17].

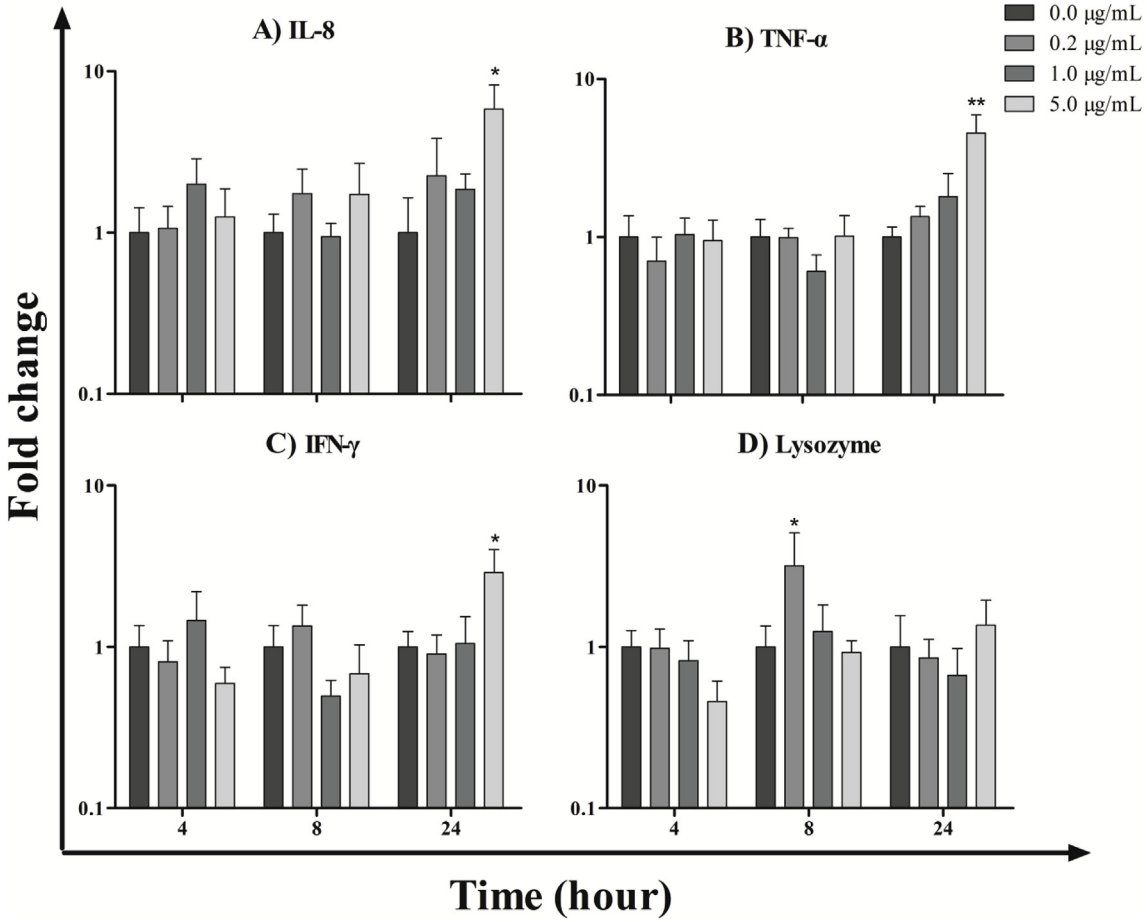

Fig. 1. Effects of F2 on IL-8, TNF-α, IFN-γ and lysozyme gene expression *in vitro*. Target gene expressions in head kidney cells were analyzed after incubating with 0 (control), 0.2, 1, 5 μg/mL of F2 for 4, 8, 24 h at 20 °C. The qRT-PCR data were normalized relative to the expression of EF-1α and fold changes were calculated by dividing the normalized ratio by control group at each time point. Data were represented as means ± SEM (n = 4) and analyzed by ANOVA post-hoc LSD test. Significant differences are marked as $*p < 0.05$ or $**p < 0.01$.

2.8. Statistical analysis

Statistical analysis was performed with SPSS 16.0 software with the data represented as the mean ± SEM of 4 or 8 fish in *in vitro* or *in vivo* assay, respectively. The homogeneity of data was checked by Levene's test, and analyzed the data by ANOVA post-hoc LSD test. The p value < 0.05 between treatment group and control group was considered to be significant.

3. Results and discussion

3.1. Effects on pro-inflammatory cytokine and lysozyme gene expression in vitro

HK cells were chosen for *in vitro* analysis since HK contains massive lymphocytes and monocytes in fish [18,19]. When they were co-incubated with F2, immune gene (IL-8, TNF-α and IFN-γ) expressions were significantly up-regulated at 24 h in 5 μg/mL group; lysozyme gene expression was significantly up-regulated at 8 h in 0.2 μg/mL group (Fig. 1). This result is in agreement with previous studies which demonstrated that F2 up-regulated inflammatory cytokine gene expressions in macrophages and NK cells via NF-κB/MAPK and CR3-mediated signaling pathways, respectively [7,8]. Since F2 is mainly composed of (1 → 3)-β-D-mannan [7], it is postulated that F2 may activate immune cells in HK through mannose-binding receptor and regulate immune functions [20].

3.2. Effects on pro-inflammatory cytokine and lysozyme gene expression in vivo

In order to analyze *in vivo* effects, immune-related tissues including PC, HK, liver, gill, and spleen were taken and found all tested gene expressions were tend to be significantly up-regulated (Figs. 2 and 3). We have previously reported the upregulated IL-1β gene expression by F2 in PC and HK [9]. This is consistent with the *in vitro* experiment, indicating an immuno-stimulatory effect of F2 in olive flounder.

On the other hand, although IL-1β and IL-8 gene expressions were up-regulated in most tissues, TNF-α, IFN-γ and lysozyme gene expressions varied depending on tissues at different time point. TNF-α was significantly up-regulated in PC and gill at day 1 and 3 while down-regulated in HK at day 3 and spleen at day 1 (Figs. 2 and 3). IFN-γ was also significantly up-regulated in PC, HK (day 1), liver and gill while down-regulated in HK at day 3 (Figs. 2 and 3). Lysozyme was significantly down-regulated in PC at day 1 but later significantly up-regulated at day 3 while it was opposite in HK (Fig. 2).

Different expressional profile of cytokine genes depending on tissues and time points may be caused by an infiltration of different cell populations in the tested organs over the time since it is known that i.p. injection of polysaccharide can induce leukocyte infiltration or variation of cell population in different tissues. For example, Currier et al. [21] found that when mice were i.p. injected with polysaccharide arabinogalactan, the immune and hemopoietic cell lineages in spleen and bone marrow varied at different time point. Indeed, other cytokines induced by SP administration may have affected TNF-α, and IFN-γ gene

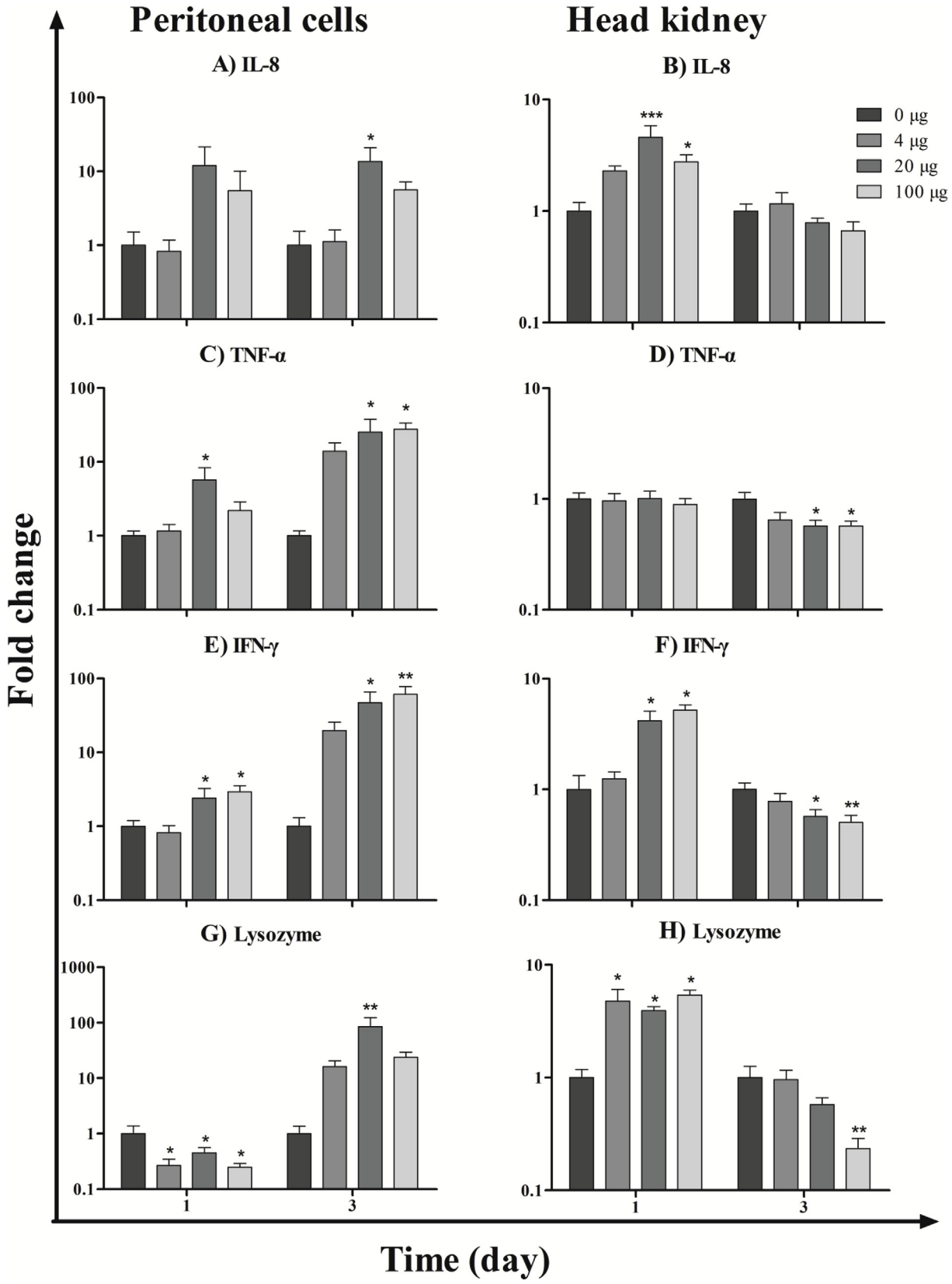

Fig. 2. Effects of F2 on IL-8, TNF-α, IFN-γ and lysozyme gene expression in PC and HK *in vivo*. Target gene expressions were analyzed in olive flounder i.p. injected with 0 (control), 4, 20 or 100 μg of F2 at 1 or 3 day. The qRT-PCR data were normalized relative to the expression of EF-1α and fold changes were calculated by dividing the normalized ratio by control group at each time point. Data were represented as means ± SEM (n = 8) and analyzed by ANOVA post-hoc LSD test. Significant differences are marked as $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$.

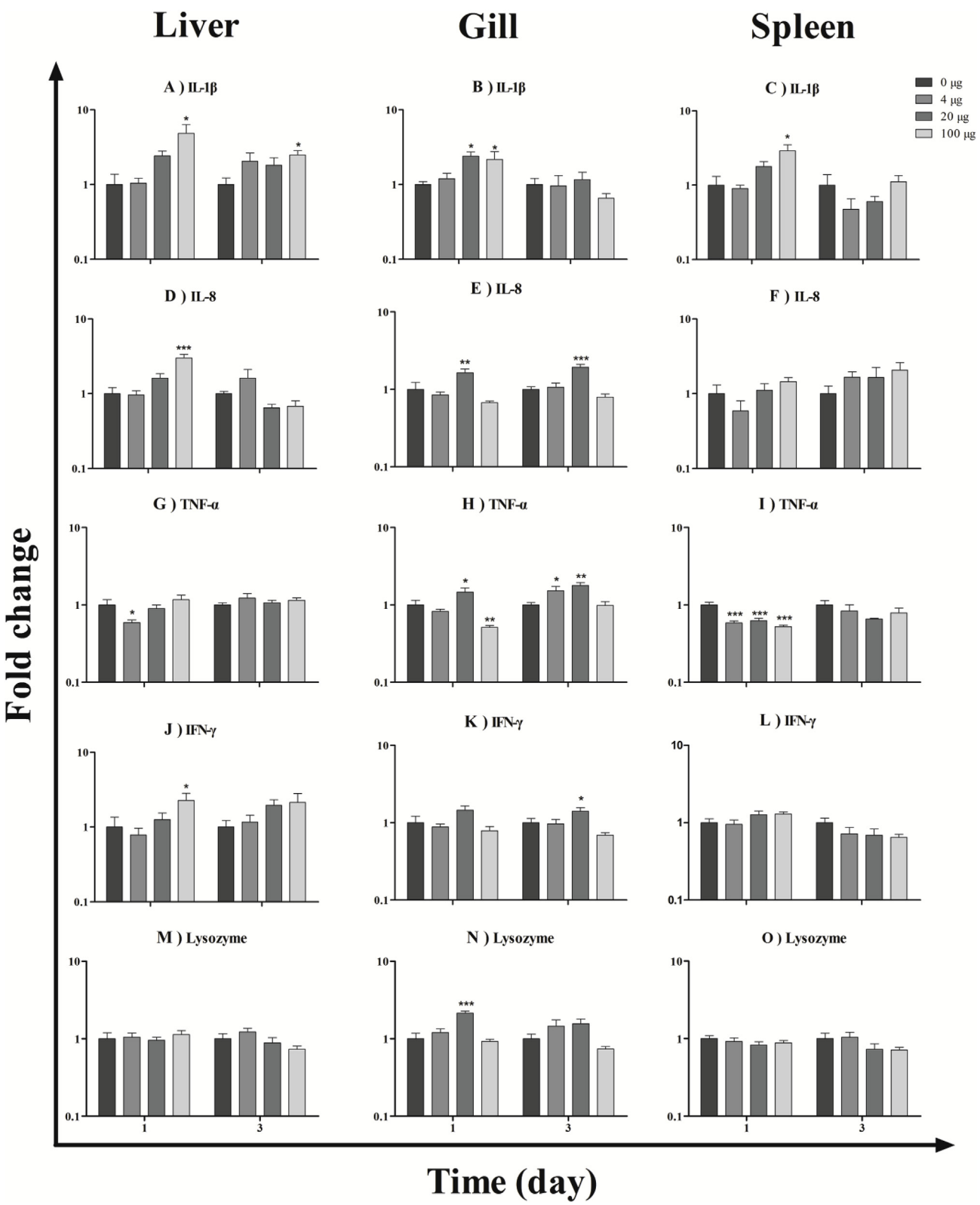

Fig. 3. Effects of F2 on IL-1β, IL-8, TNF-α, IFN-γ and lysozyme gene expression in liver, gill and spleen *in vivo*. Target gene expressions were analyzed in olive flounder i.p. injected with 0 (control), 4, 20 or 100 μg of F2 at 1 or 3 day. The qRT-PCR data were normalized relative to the expression of EF-1α and fold changes were calculated by dividing the normalized ratio by control group at each time point. Data were represented as means ± SEM (n = 8) and analyzed by ANOVA post-hoc LSD test. Significant differences are marked as $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$.

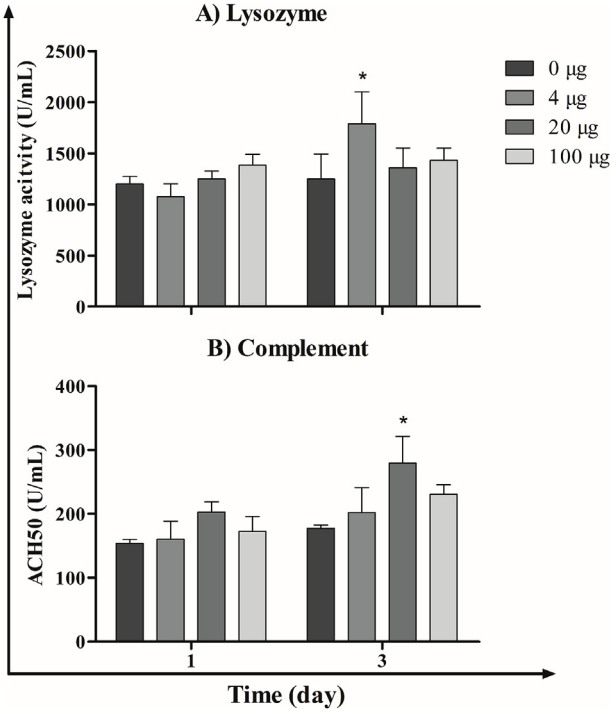

Fig. 4. Effects of F2 on serum lysozyme and complement activity. Lysozyme (A) and complement (B) activity was analyzed in serum of olive flounder i.p. injected with 0 (control), 4, 20, 100 μg of F2 at 1 or 3 day. Data were represented as means ± SEM (n = 8) and analyzed by ANOVA post-hoc LSD test. Significant differences are marked as $*p < 0.05$.

expression in this study. In previous mammalian study, F2 was found to up-regulate IL-6 and IL-10 [7] which are known to have anti-inflammatory function and down-regulate TNF-α and/or IFN-γ [22–25]. On the other hand, it is also known that various transcription factors oscillate over time and the oscillation can induce different cytokine gene expression pattern [26].

3.3. Effects on serum lysozyme and complement activity

In the humoral assay, lysozyme and complement activities were assessed. The lysozyme activity in serum was remarkably up-regulated in 4 μg group at day 3 (Fig. 4A). According to Sakthivel et al. [27], when Asian seabass (*Lates calcarifer*) was fed with polysaccharide from *Kappaphycus alvarezii*, an enhanced lysozyme activity was observed; however, when i.p. injected with the same polysaccharide, the activity was down-regulated. Peddie et al. [28] observed up-regulation of pro-inflammatory cytokine genes expression when rainbow trout (*Oncorhynchus mykiss*) was i.p. injected with Ergosan, a commercial algine based complimentary feedstuff for fish and crustaceans, but lysozyme activity was unaffected. This could indicate lysozyme activity of olive flounder is only limited to a narrow F2 dose range.

Meanwhile, in this study, alternative complement activity was significantly up-regulated in 20 μg group at day 3 (Fig. 4B), indicating that F2 can enhance the complement activity. Complement system is a vital component of the innate immunity system as it can kill the invading pathogens and serve as a link between innate and adaptive immunity [29].

In conclusion, the F2 of SPs from *C. fragile* can stimulate the expression of IL-1β, IL-8, TNF-α, IFN-γ, and lysozyme genes in major immune organs and enhance the lysozyme and complement activities in serum of olive flounder, indicating that the F2 has a similar immunostimulating effect on fish as on mammalian cells and could enhance the immune protection in fish. Future work will be focused to confirm its effects on the feeding trial. If the feeding trial obtains similar results, the polysaccharides from *C. fragile* may be used as feeding additives to enhance the immunity of fish.

Acknowledgements

This research was supported by Marine Bio-regional Specialization Leading Technology Development Program (D11413914H-480000100) funded by the Ministry of Oceans and Fisheries, Korea.

References

[1] Ministry of Oceans and Fisheries (MOF), (2018) http://www.mof.go.kr/content/view.do?menuKey=39.4&contentKey=47 , Accessed date: 13 February 2018.
[2] D.P. Anderson, Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture, J. Fish. Dis. 2 (1992) 281–307.
[3] D.H. Ngo, S.K. Kim, Sulfated polysaccharides as bioactive agents from marine algae, Int. J. Biol. Macromol. 62 (2013) 70–75.
[4] L.S. Costa, G.P. Fidelis, S.L. Cordeiro, R.M. Oliveira, D.A. Sabry, R.B.G. Camara, L.T.D.B. Nobre, M.S.S.P. Costa, J. Almeida-Lima, E.H.C. Farias, E.L. Leite, H.A.O. Rocha, Biological activities of sulfated polysaccharides from tropical seaweeds, Biomed. Pharmacother. 64 (2010) 21–28.
[5] M. Tabarsa, J.H. Han, C.Y. Kim, S.G. You, Molecular characteristics and immunomodulatory activities of water-soluble sulfated polysaccharides from *Ulva pertusa*, J. Med. Food 15 (2012) 135–144.
[6] J. Provan, S. Murphy, C.A. Maggs, Tracking the invasive history of the green alga *Codium fragile* ssp. *tomentosoides*, Mol. Ecol. 14 (2005) 189–194.
[7] M. Tabarsa, S. Karnjanapratum, M.L. Cho, J.K. Kim, S.G. You, Molecular characteristics and biological activities of anionic macromolecules from *Codium fragile*, Int. J. Biol. Macromol. 59 (2013) 1–12.
[8] U. Surayot, S.G. You, Structural effects of sulfated polysaccharides from *Codium fragile* on NK cell activation and cytotoxicity, Int. J. Biol. Macromol. 98 (2017) 117–124.
[9] Y. Yang, S.G. You, S. Hong, Immuno-stimulatory Effects of sulfated polysaccharides isolated from *Codium fragile* on Interleukin-1β gene expression in olive flounder, *Paralichythys olivaceus*, J. Marine Life Sci. 2 (2017) 1–5.
[10] J. Zou, C.J. Secombes, The function of fish cytokines, Biology 5 (2016) 23.
[11] M. Bickel, The role of interleukin-8 in inflammation and mechanisms of regulation, J. Periodontol. 64 (1993) 456–460.
[12] J.R. Schoenborn, C.B. Wilson, Regulation of Interferon-γ during innate and adaptive immune responses, Adv. Immunol. 96 (2007) 41–101.
[13] L. Callewaert, C.W. Michiels, Lysozymes in the animal kingdom, J. Biosci. 35 (2010) 127–160.
[14] R.M. Jiménez-Cantizano, C. Infante, B. Martin-Antonio, M. Ponce, I. Hachero, J.I. Navas, M. Manchado, Identification and expression analysis of the g-type and c-type lysozymes in grass carp *Ctenopharyngodon idellus*, Fish Shellfish Immunol. 25 (2008) 57–65.
[15] X. Ye, L. Zhang, Y. Tian, A. Tan, J. Bai, S. Li, Molecular characterization, phylogeny, and expression of c-type and g-type lysozymes in brill (*Scophthalmus rhombus*), Dev. Comp. Immunol. 34 (2010) 501–509.
[16] T. Yano, H. Matsuyama, R.E.P. Mangindaan, Polysaccharide-induced protection of carp, *Cyprinus carpio* L., against bacterial infection, J. Fish. Dis. 14 (1991) 577–582.
[17] M. Bagni, L. Archetti, M. Amadori, G. Marino, Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*), J. Vet. Med. 47 (2000) 745–751.
[18] L. Taysse, C. Chambras, D. Marionnet, C. Bosgiraud, P. Deschaux, Basal level and induction of cytochrome P450, EROD, UDPGT, and GST activities in carp (*Cyprinus carpio*) immune organs (spleen and head kidney), Bull. Environ. Contam. Toxicol. 60 (1998) 300–305.
[19] E. Kondera, Cell composition of the head kidney of European chub (*Squalius cephalus* L.), Arch. Pol. Fish. 22 (2014) 271–280.
[20] L. Cunha, A. Grenha, Sulfated seaweed polysaccharides as multifunctional materials in drug delivery applications, Mar. Drugs 14 (2016) 42.
[21] N. Currier, D. Lejtenyi, S. Miller, Effect over time of *in-vivo* administration of the polysaccharide arabinogalactan on immune and hemopoietic cell lineages in murine spleen and bone marrow, Phytomedicine 10 (2003) 145.
[22] L. Shapiro, C.A. Dinarello, Hyperosmotic stress as a stimulant for proinflammatory cytokine production, Exp. Cell Res. 231 (1997) 354–362.
[23] Z. Xing, J. Gauldie, G. Cox, H. Baumann, M. Jordana, X.F. Lei, M.K. Achong, IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses, J. Clin. Invest. 101 (1998) 311–320.
[24] D.I. Shin, U. Banning, Y.M. Kim, J. Verheyen, M. Hannen, H. Bönig, D. Körholz, Interleukin 10 inhibits TNF-alpha production in human monocytes independently of interleukin 12 and interleukin 1 beta, Immunol. Invest. 28 (1999) 165–175.
[25] A. D'Andrea, M. Aste-Amezaga, N.M. Valiante, X. Ma, M. Kubin, G. Trinchieri, Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells, J. Exp. Med. 178 (1993) 1041–1048.
[26] K.B. Wee, W.K. Yio, U. Surana, K.H. Chiam, Transcription factor oscillations induce differential gene expressions, Biophys. J. 102 (2012) 2413–2423.
[27] M. Sakthivel, B. Deivasigamani, T. Rajasekar, S. Kumaran, K. Alagappan, Immunostimulatory effects of polysaccharide compound from seaweed *Kappaphycus alvarezii* on Asian seabass (*Lates calcarifer*) and it's resistance against *Vibrio parahaemolyticus*, J. Mar. Biol. Oceanogr. 4 (2015) 2.
[28] S. Peddie, J. Zou, C.J. Secombes, Immunostimulation in the rainbow trout (*Oncorhynchus mykiss*) following intraperitoneal administration of Ergosan, Vet. Immunol. Immunopathol. 86 (2002) 101–113.
[29] M. Claire, H. Holland, J.D. Lambris, The complement system in teleosts, Fish Shellfish Immunol. 12 (2002) 399–420.